Development of Taqman RT-nested PCR system for clinical SARS-CoV detection

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Abstract

Severe acute respiratory syndrome (SARS) is an acute newly emerged infectious respiratory illness. The etiologic agent of SARS was named 'SARS-associated coronavirus' (SARS-CoV) that can be detected with reverse transcription-polymerase chain reaction (RT-PCR) assays. In this study, 12 sets of nested primers covering the SARS-CoV genome have been screened and showed sufficient sensitivity to detect SARS-CoV in RNA isolated from virus cultured in Vero 6 cells. To optimize further the reaction condition of those nested primers sets, seven sets of nested primers have been chosen to compare their reverse transcribed efficiency with specific and random primers, which is useful to combine RT with the first round of PCR into a one-step RT-PCR. Based on the sensitivity and simplicity of results, the no. 73 primer set was chosen as the candidate primer set for clinical diagnoses. To specify the amplicon to minimize false positive results, a Taqman RT-nested PCR system of no. 73 nested primer set was developed. Through investigations on a test panel of whole blood obtained from 30 SARS patients and 9 control persons, the specificity and sensitivity of the Taqman RT-nested PCR system was found to be 100 and 83%, respectively, which suggests that the method is a promising one to diagnose SARS in early stages.
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1. Introduction

An outbreak of a newly emergent infectious disease referred to as severe acute respiratory syndrome (SARS), first identified in Guangdong Province, China in November 2002, infected as many as 8436 individuals and resulted in 812 deaths around the world by 9 July 2003 (http://www.who.int/csr/sars/en/). The etiologic agent of SARS has been identified as a new coronavirus and named as the SARS-associated coronavirus (SARS-CoV) (Peiris et al., 2003; Ksiazek et al., 2003; Drosten et al., 2003).

SARS-CoV is an enveloped, positive-strand RNA virus. Genomes of more than 17 strains of SARS-CoV have been sequenced (Marra et al., 2003; Rota et al., 2003; Ruan et al., 2003; Qin et al., 2003). The size of genome is about 29–30 kb, which is the longest RNA positive strand virus to date. Based on the genetic analysis of the genome sequence, the SARS-CoV has been classified as a distinct group from any previously known coronavirus (Drosten et al., 2003).

The symptoms of SARS resemble those of other forms of 'atypical pneumonia' that are usually caused by mycoplasma, *Chlamydia* species, and others. In the absence of effective drugs and vaccines for SARS, rapid identification of this disease is of importance for controlling spread. At present, virus isolation by cell culture, ELISA, IFA and reverse transcription-polymerase chain reaction (RT-PCR) are the major methods to diagnose SARS. ELISA and IFA methods depend on the detection of IgG and/or IgM against SARS-CoV virus that emerged in the blood during the later phase of infection (Ksiazek et al., 2003; Drosten et al., 2003). There is a critical need for a test that can detect SARS at early stages of illness (days 1–3) since persons infected with the SARS-CoV can infect a large number of individuals easily (Rosling and Rosling, 2003). The detection of

viral RNA with RT-PCR assays is the most widely used approach for early detection of pathogens (Drosten et al., 2003; Poon et al., 2003). Based on the genomic sequence of BJ01 (accession number: AY278488), 12 sets of nested primers covering SARS-CoV have been synthesized. In this study a methodology for selecting the no. 73 nested primers as the most promising primers for clinical purposes and a Taqman RT-nested PCR system using the no. 73 nested primer set are described, and the system was evaluated on a test panel.

2. Material and methods

2.1. Primer sets and fluoregenic probe

The primers were designed with the genome of BJ01 strain of SARS-CoV (AY278488) as the target. Each set of primers was designed with Primer3 software freely available through internet (http://www.basic.northwestern.edu/bio tools/Primer3.html). The size of outer amplicon and inner amplicon are 200–280 and 100–140 bp, respectively. The annealing temperature was restricted at the range of 52–56 °C. The primer sequences are summarized in Table 1.

These primers were synthesized by a DNA Biotechnology Company (Shanghai).

The fluoregenic probe for no. 73 nested primer sets 5′-FAM-*aag gtg aca ctc gct gat gct g*-TAMRA-3′ was provided by Sangon Biotechology Company (Shanghai). FAM represents the fluorescent reporter, and the TAMRA quencher is linked with the last nucleotide G.

2.2. RNA isolation

The RNA of viruses cultured in Vero 6 cell was extracted with the QIAamp viral RNA Mini Kit (Qiagen). Whole blood samples of 30 patients diagnosed clinically with SARS were used in the panel. All patients (female/male = 11/19, age = 32.8 ± 11.9) had high fever, and the day with the highest temperature was regarded as day 1. In the meantime, whole blood samples of nine control persons were also included in the test panel. The QIAamp RNA Blood Mini Kit (Qiagen) was used to prepare the whole blood samples of the test panel. According to the manufacture's instructions, RNA was eluted in a final volume of 60 μl of elution buffer. All the above procedures were performed in biosafety level 3 laboratory.

Table 1
The summary of 12 sets of nested PCR primers used in this study

Nos.	Outer primer		Size (bp)	Inner primer		Size (bp)	Genome position
		Nucleotide sequence			Nucleotide sequence		
9	F	TCCTGGTTTACTGGCTACAA	238	F	AGAATCACATTTGAGCTTGATG	103	R1a
	R	CTTCACAACAGCCTCTGCTA		R	CACATGCAAACTCAGTAACTTC		
14	F	GCAAACCTGTGCCATATTGT	279	F	GTTATGTGCTGATGGATGGTTC	150	R1a
	R	CACAGAAAACTCCTGATAGAGC		R	ACCACTGGTAGATAGGCAAATAC		
62	F	TATAAGCTCGAGGGCTATGC	180	F	GACAACTTGGCGGTCTTC	100	R1b
	R	TTGCGCATCTGTTATGAAGT		R	TTCACTGTGCTGTCCATAGG		
73	F	CAAAACCCCAACTTTGAAAT	226	F	TCTTTTATTGAGGACTTGCTCT	118	S
	R	CAGAGGTGGCAACACTGTAA		R	ACTTCTGCGCACAAATGAG		
75	F	CTAATCAGGGCTGCTGAAAT	248	F	GAAAGGGCTACCACCTTATG	101	S
	R	ACACCTTCACGAGGGAAGTA		R	TGTGGTGAAGTTCCTCTCCT		
79	F	CTGAGGATAGGCACTCAGGT	181	F	CATGGCTATTTCACCGAAGT	100	PUP1
	R	CCGTCGATTGTGTGTATTTG		R	CAAGCTTGTTAAAGATGAAGAA		PUP2
80	F	TTTCGGAAGAAACAGGTACG	277	F	CACACTAGCCATCCTTACTGC	105	E
	R	TCTGCCATGATAAGCAATGT		R	TTTAACACGCGAGTAGACGTA		
81	F	AACTCCTGGAACAATGGAAC	238	F	TTCCTAGCCTGGATTATGTT	120	M
	R	TAAGCCACATCAAGCCTACA		R	AGCACAAAACAAGCAAGTGT		
83	F	TGAGGACTTTCAGGATTGCTA	254	F	ATTCGGAGTTAGATGATGAAGA	100	PUF3
	R	GATGGGCAAGGTTCTTTTAG		R	CGCAAGATGTAAATACAATCAA		
86	F	GATAATGGACCCCAATCAAA	268	F	CCCACAGATTCAACTGACAA	102	N
	R	ATTTGGTCATCTGGACCACT		R	TGTGAACCAAGACGCAGTAT		
87	F	AGCCCCAGATGGTACTTCTA	246	F	AACTGAGGGAGCCTTGAATA	101	N
	R	AATTACCGCGACTACGTGAT		R	GGCAATGTTGTTCCTTGAG		
90	F	GGCAGATGGGCTATGTAAAC	279	F	TGCAGAATGAATTCTCGTAACT	150	3′ UTR
	R	TTCCATATAGGCAGCTCTCC		R	CAAGTCCTCCCTAATGTTACAC		

2.3. Reverse transcription

The reverse transcription reaction was carried out separately with a random primer and a specific primer. Each reaction mixture includes 100 ng random primers or 1 µl of 10 µm specific primer, 10 µl of total RNA and 1 µl dNTP (10 mM). The mixture was heated at 95 °C for 3 min and then placed immediately on ice. The following reagent was then added to the mixture: 4 µl of first strand-synthesis buffer (5×, Promega), 2 µl of 0.1 M DTT (Promega), 1 µl RNase Inhittor (40 u/µl, Promega) and 1 µl of SuperScript II RNase H-RT (200 u/µl, Invitrogen). The RT reaction was carried out at 25 °C for 10 min, followed by incubation at 42 °C for 40 min, and then finally inactivation of the enzyme at 70 °C for 15 min.

2.4. PCR amplification with nested PCR

The first round PCR was carried out in a 25 µl volume containing 2 µl of RT product, 0.2 µM of each primer, 1 unit of Taq DNA polymerase (Promega), 0.1 mM dNTPs, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.25 mM MgCl$_2$. Thermocycling was performed on a thermocyler of MJ-research PTC-200 (MJ-research Inc.), with initial denaturation for 2 min at 95 °C, followed by 35 cycles of (95 °C for 30 s, optimal Tm for 30 s, 72 °C for 40 s). The final extension reaction was performed for 2 min at 72 °C to complete all the PCR reactions.

The second round of PCR was on undertaken on iCycler (Bio-Rad). To detect the PCR product, SYBR Green I or Taqman probe in the second round reaction was used in the mixture. The reaction volume for the second round of PCR was also 25 µl, containing 2 µl product of first round PCR reaction, 0.2 µM of each primer, 1 unit of Taq DNA polymerase (Promega), 0.1 mM dNTPs, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.25 mM MgCl$_2$ and 1.25 µl of SYBR Green I or 0.5 µl of Taqman probe (5 µM). For using the Taqman probe in mixture, thermocycling was carried out with initial denaturation for 2 min at 95 °C, followed by 40 cycles of (95 °C for 30 s, 60 °C for 1 min). For detection with SYBR Green I, thermocycling was carried out with initial denaturation for 2 min at 95 °C, followed by 35 cycles of (95 °C for 30 s, optimal Tm for 15 s, 72 °C for 15 s). A melting curve was completed from 55 to 95 °C, increasing by 0.5 °C per cycle, to investigate the amplicons.

3. Result

3.1. The sensitivities of 12 sets of nested primers

The 12 sets of nested primers used in the study distributed unevenly in the SARS genome, but they spanned all known genes and the two predicted genes identified in the SARS-CoV genome (Fig. 1). To compare the sensitivities of these 12 sets of nested primers, serial 10-fold di-

lution genome cDNA of BJ01 that reverse transcribed with random primer was used as the template to carry out the nested PCR. The initial concentration of total RNA from virus and Vero 6 cells was 50 ng/µl, which corresponds to $<10^{13}$ molecules of SARS-CoV per microliter. All nested primers sets could detect up to 10^{-10} diluted RNA, which corresponds to 10^2–10^3 copies of SARS genome per microliter. For nos. 73, 79, and 80 nested primers sets, several copies of SARS genome could be detected. Although few sporadic signals occurred for diluted templates from 10^{-13} to 10^{-15}, they represent random, unreliable results (Fig. 2).

3.2. The efficiency of reverse transcribed with random primer versus specific primer

To compare the reverse transcribed efficiency of random primer versus specific primer, seven sets of nested primers covering each known gene were chosen for testing. With the 10-fold diluted BJ01 genome RNA series, the reverse transcriptions were carried out separately with a random primer and a specific primer. Then, amplification with outer primer on the cDNA was performed. The products were monitored with SYBR Green I that binds with double DNA. The results showed that three out of seven sets of nested primers have better efficiency on templates reverse transcribed with the specific primer, and three other sets of primers showed that the reverse transcription with the random primer has higher efficiency than with the specific primer. It is worthy to note that the four sets of nested primers located on the terminus of the genome have higher efficiency when transcribed with the random primer. In contrast, the other three sets of primers targeting the initial region of genome have higher efficiency when transcribed with the specific primer. The outer reverse primer pairs of no. 73 are the most sensitive on cDNA reverse transcribed with specific primer. Meanwhile, the no. 80 primers can attain the same sensitivity on the cDNA reverse transcribed with the random primers (Fig. 3).

3.3. The specificity of the Taqman RT-nested PCR system

As the no. 73 nested primers set were found to be the most promising primers for clinical diagnosis, a Taqman probe targeting the internal region of the no. 73 inner amplicon has been designed to minimize false positive results. The Taqman probe and no. 73 nested primers pairs together constitute a Taqman RT-nested PCR system. To test the specificity of the Taqman RT-nested PCR system, a panel consisting of 30 sets of RNA from SARS patients' whole blood and 9 sets of RNA from control person's whole blood was used. These RNA samples were reverse transcribed with the no. 73 outer reverse primer. After the first round of PCR, a second round of PCR was performed and monitored with real time PCR. None of the control samples produced a signal, whereas, 25 of the 30 samples from SARS patients produced a detectable signal. The results showed the sensitivity and
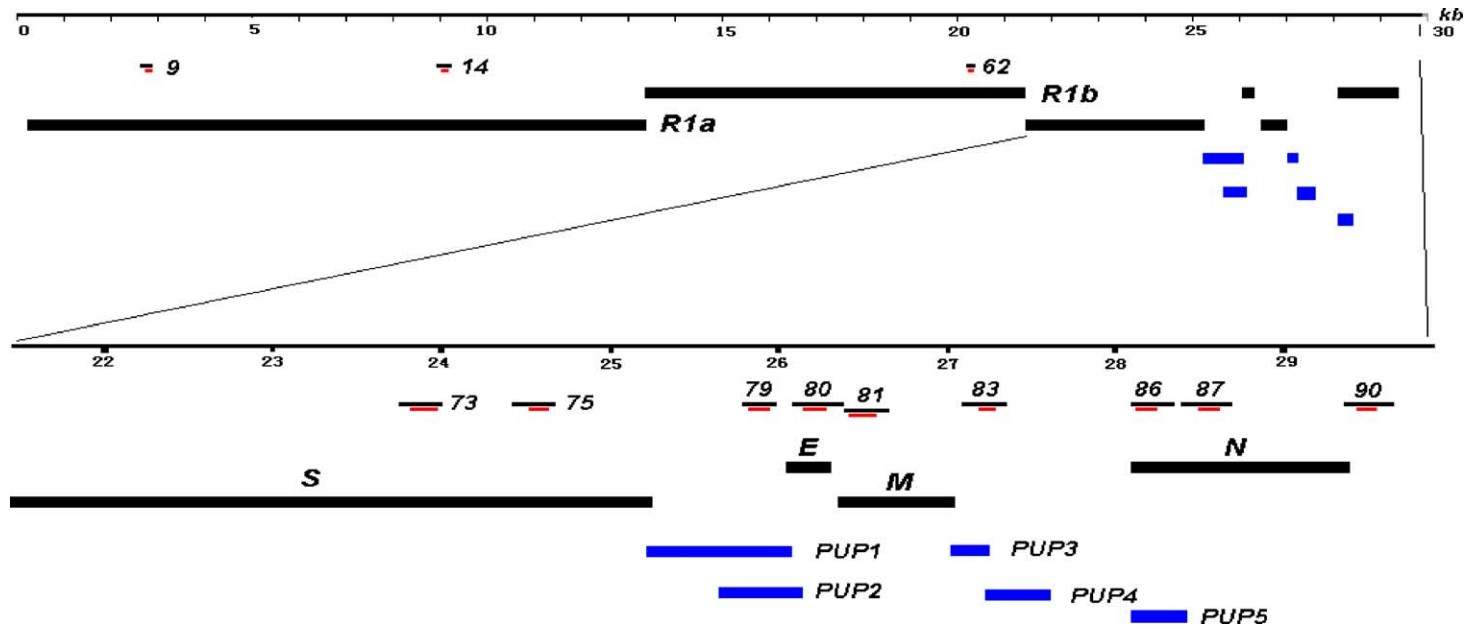
Fig. 1. The distribution of 12 sets of nested primers across the genome of BJ01 (AY278488). The black bars represent known genes, the blue bars represent predicted genes. The 12 sets of nested primers are also shown in the map. The black or longer line represents the region of outer amplicon, whereas the red or shorter line represents the inner amplicon. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

	9	14	62	73	75	79	80	81	83	86	87	90
1X	★	★	★	★	★	★	★	★	★	★	★	★
10^{-1}	★	★	★	★	★	★	★	★	★	★	★	★
10^{-2}	★	★	★	★	★	★	★	★	★	★	★	★
10^{-3}	★	★	★	★	★	★	★	★	★	★	★	★
10^{-4}	★	★	★	★	★	★	★	★	★	★	★	★
10^{-5}	★	★	★	★	★	★	★	★	★	★	★	★
10^{-6}	★	★	★	★	★	★	★	★	★	★	★	★
10^{-7}	★	★	★	★	★	★	★	★	★	★	★	★
10^{-8}	★	★	★	★	★	★	★	★	★	★	★	★
10^{-9}	★	★	★	★	★	★	★	★	★	★	★	★
10^{-10}	★	★	★	★	★	★	★	★	★	★	★	★
10^{-11}				★	★	★	★	★	★		★	★
10^{-12}				★		★	★					
10^{-13}								★				
10^{-14}				★								
10^{-15}												★
10^{-16}												
10^{-17}												

Fig. 2. The schematic map of sensitivities of 12 sets of nested primers tested on serial 10-fold diluted templates. The initial concentration of total RNA from virus and Vero 6 cells is 50 ng/μl, which corresponds to $<10^{13}$ molecules of SARS-CoV per microliter. The asterisk represents positive signal. The sensitivities of nos. 73, 79, and 80 are 100–1000-fold greater than other nested primers pairs.
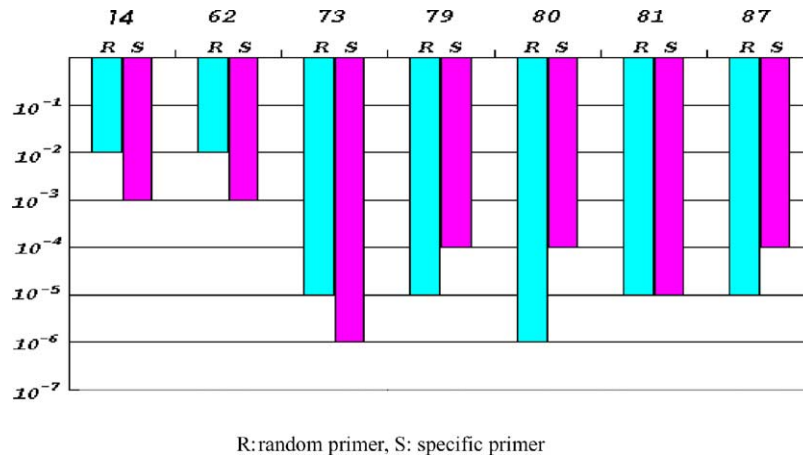

R: random primer, S: specific primer

Fig. 3. Comparative efficiencies of reverse transcription primed by the random primer or a specific primer on 10-fold diluted series of BJ01 genome. The lower margin of each bar shows the level of sensitivity of these outer primers. The nos. 14, 62, and 73 have higher reverse transcription efficiency when transcribed with specific primer, whereas those primers of nos. 79, 80, 81, and 87 have higher reverse transcription efficiency when transcribed with the random primer. The values on vertical axis represent the diluted concentration of RNA compared with that of initial RNA (50 ng/μl).

the specificity of the Taqman RT-nested PCR system to be 83 and 100%, respectively (Table 2).

4. Discussion

The most striking features of the SARS-associated virus are its spikes on the sphere-like virion, which can be clearly observed under the electron microscope and are the basis of the name 'coronavira'. The spike (S) protein, together with the E and M proteins, constitute the components of the surface of coronaviruses. The replicase (R) protein, which occurs in limited amounts, including 1a and 1b is the defined unique non-structural protein responsible for RNA replication. According to the Northern blot, the subgenomic regions for S, M, and E are transcribed in larger quantities (Rota et al., 2003). With the 10-fold diluted RNA as template, the sensitivity of primers targeting the S, M, E, and N proteins is higher than 10–100-fold than those primers targeting the R protein, which suggests that the sensitivity of detection is determined mostly by the quantity of transcripts.

Nucleic acid amplification techniques combining reverse transcription and the polymerase chain reaction have been applied to the detection of SARS. The primer is the primary component necessary for developing RT-nested PCR. The size of outer amplicon was set range from 200 to 280 bp, which were usually sufficiently long to design inner primers with spacing intervals of ∼100 bp. Compared with longer PCR products, this range and flexibility enables many assays to be produced successfully. For these primers, the reverse transcriptions with random primer and specific primer have different effects. According to the sensitivity and simplicity of our test result, the no. 73 primer appears to be the most promising primers set for clinical diagnosis, which is very

Table 2
The specificity of the Taqman RT-nested PCR system on tested panel

Case	Sex	Age	Day from onset	Patient with history of RT-PCR					RT-PCR result in this study	Comment
				Contact history	High fever (>38 °C)	Leukocyte count ($\times 10^9$/ml)	Cough or breathing difficulty	Evidence of pulmonary infiltrates		
1	M	54	4	Y	Y	6.8	Y	Y	Y	Confirmed
2	F	24	6	Y	Y	7.7	Y	Y	Y	SARS
3	M	22	9	Y	Y	7.5	Y	Y	Y	patients[a]
4	M	22	9	Y	Y	7.5	Y	NA	Y	
5	M	27	9	Y	Y	5.9	Y	NA	Y	
6	M	25	10	Y	Y	4.3	Y	NA	N	
7	M	40	10	Y	Y	8.1	Y	Y	Y	
8	M	20	12	Y	Y	5.3	Y	Y	Y	
9	M	23	13	Y	Y	6.2	Y	Y	Y	
10	M	36	13	Y	Y	2.7	Y	Y	Y	
11	F	21	14	Y	Y	4.9	Y	NA	Y	
12	F	38	14	Y	Y	2.6	Y	Y	Y	
13	M	24	14	Y	Y	5.3	Y	Y	Y	
14	F	32	15	Y	Y	7.4	Y	N	'1,	
15	F	49	15	Y	Y	4.9	Y	Y	Y	
16	F	29	15	Y	Y	3.1	Y	Y	Y	
17	F	66	16	Y	Y	4.3	Y	Y	Y	
18	M	47	16	Y	Y	5.3	Y	NA	Y	
19	F	52	16	Y	Y	5.1	Y	Y	Y	
20	M	23	16	Y	Y	4.7	Y	Y	N	
21	F	10	16	Y	Y	2.4	Y	Y	N	
22	M	38	17	Y	Y	4.7	Y	Y	Y	
23	M	30	18	Y	Y	8.3	Y	Y	N	
24	M	21	18	Y	Y	5.3	Y	Y	Y	
25	F	28	18	Y	Y	4.8	Y	Y	Y	
26	M	47	18	Y	Y	2.9	Y	Y	Y	
27	M	38	19	Y	Y	4.8	Y	Y	Y	
28	F	29	19	Y	Y	5.7	Y	Y	Y	
29	M	33	19	Y	Y	3.8	Y	Y	Y	
30	M	30	20	Y	Y	5.1	Y	NA	Y	
31	M	18	–	N	N	NA	N	NA	N	Control
32	F	22	–	N	N	NA	N	NA	N	group
33	F	21	–	N	N	NA	N	NA	N	
34	F	21	–	N	N	NA	N	NA	N	
35	M	22	–	N	N	NA	N	NA	N	
36	M	20	–	N	N	NA	N	NA	N	
37	F	23	–	N	N	NA	N	NA	N	
38	F	19	–	N	N	NA	N	NA	N	
39	M	22	–	N	N	NA	N	NA	N	

NA: not available, Y: yes, N: no.

[a] These SARS patients were confirmed by the ELISA assay 2–4 weeks after onset (data not shown).

useful to simplify the detection process through combining RT and first round of PCR reactions to one-step RT-PCR reaction.

Because the nested PCR can detect low amounts of virus, it is necessary and important to adopt strict criteria for confirmation of positive results. The product can be confirmed as SARS-CoV by a number of other methods including Taqman, DNA chip, PCR–ELISA, and sequencing. In this study, the Taqman probe was applied to confirm the PCR product. The Taqman system was developed by ABI and employs a fluoregenic probe-based 5′, exonuclease technology that enables amplification and detection to be carried out simultaneously, eliminating the need for post-PCR analysis (Heid et al., 1996; Fortin et al., 2001). Use of the fluorescent probe

in real time PCR provides an additional level of assay specificity. Although fluorescence increases in direct proportion to the amount of specific amplicons, the Taqman probe was used to specify the product in this study because the fluorescence increases non-linearly in the second round of amplification compared with the original virus RNA concentration. To confirm positive amplicon, the PCR procedure should include appropriate negative and positive controls in each run, including negative and positive controls for the extraction procedure and the PCR run.

SARS-CoV has been detected in multiple specimens including extracts of lung, sputum, upper respiratory tract swabs, aspirate, stool, and blood samples via PCR or viral isolation (Ksiazek et al., 2003; Drosten et al., 2003; Poon

et al., 2003). High concentration of viral RNA of up to 100 million molecules per milliliter has been detected in sputum. Viral RNA has also been detected at extremely low concentrations in plasma during the acute illness phase and in feces during the late convalescent phase, suggesting that the SARS-CoV may be shed in feces for prolonged periods of time (Drosten et al., 2003). Compared with sputum and feces, blood is used more frequently in clinical practice because it is relatively easy to obtain. In this study, whole blood was therefore used as test sample in order to develop a clinically useful detection method for the early stage of infection. According to the protocol developed in the study, the sensitivity and specificity of the Taqman RT-nested PCR system are 83 and 100%, respectively. Negative results cannot exclude the existence of SARS-CoV in tested samples, which may be attributed to low amounts of RNA in the samples. The sensitivity of RT-nested PCR tests for SARS depends on the specimen and the time of testing during the course of the illness.

Rapid diagnosis not only offers considerable benefits when a positive case is quickly identified, but is also equally informative when rapid negative result are obtained for optimal patient management and appropriate therapy. The Taqman RT-nested PCR system described here provides a rapid, sensitive, and cost effective approach for the diagnosis of SARS-CoV infection.

Acknowledgements

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